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(71) Applicant:
**CHUGAI SEIYAKU KABUSHIKI KAISHA**
**Tokyo, 115-8543 (JP)**

(72) Inventors:
- **KATO, Yukio**
  **Hiroshima-shi, Hiroshima 732-0062 (JP)**
- **KAWAMOTO, Takeshi**
  **Yoshiki-gun, Yamaguchi 754-1200 (JP)**

(74) Representative: **HOFFMANN - EITLE**
**Patent- und Rechtsanwälte**
**Arabellastrasse 4**
**81925 München (DE)**

# (54) GENE ORIGINATING IN HUMAN CHONDROCYTE

(57) This invention provides a gene specifically expressed in differentiated human chrondrocytes. By culturing the chrondrocytes in the presence of dibutyryl cAMP, the chrondrocytes are cultured in a differentiated state and any gene is searched for which has a distinction in expression between differentiated chrondrocytes and dedifferentiated chrondrocytes. This allows the gene specifically expressed in the former to be obtained.

*Fig.2A*

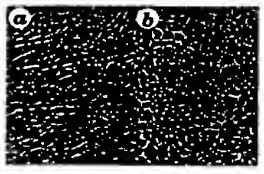
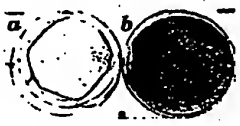



*Fig.2B*

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## Description

### TECHNICAL FIELD

[0001] This invention relates to a gene expressed specifically in differentiated chondrocytes originating in human (or human chondrocytes), a protein encoded by the gene, an antibody capable of binding to the protein, a method for culturing human chondrocytes in a differentiated state, and human chondrocytes that have been cultured by the method.

### BACKGROUND ART

[0002] Searching for genes expressed specifically in chondrocytes in a differentiated state and analysis of the properties of the chondrocytes are not only important in analyzing the mechanism of differentiation and degeneration of cartilage, but also are indispensable for developing gene therapy for osteoarthritis and rheumatoid arthritis.

[0003] However, any method for monolayer culturing human chondrocytes in a differentiated state has not yet been established, although culture systems for rabbit or chicken chondrocytes in a differentiated state have been developed. (Kato et al. Proc. Natl. Acad. Sci. USA 85, 9552-9556 (1988); Oakes et al. J. Embryol. Exp. Morphol. 38, 239-263 (1977).) It is recognized that human chondrocytes maintain their differentiated phenotype in agarose gel (Benya P.D. and Shaffer J.D., Cell 30, 215-224 (1982)), but that they easily lose the differentiated phenotype in the monolayer culture which facilitates handling of cells. Accordingly, it is difficult to search for genes that are expressed specifically in human chondrocytes in a differentiated state thereof; there has not been provided any cell culture system useful in analyzing the properties of the chondrocytes in a differentiated state thereof.

### DISCLOSURE OF INVENTION

[0004] A principle object of this invention is to establish a method of monolayer culture for human chondrocytes in a differentiated state and further to obtain a gene expressed specifically in the chondrocytes in a differentiated state thereof.

[0005] The present inventors found that chondrocytes could be cultured in a differentiated state by culturing the chondrocytes in the presence of a certain compound; and in addition, they searched for genes having a distinction in expression between differentiated chondrocytes and dedifferentiated chondrocytes and discovered those which were specifically expressed in the former. Thus, this invention has been accomplished.

[0006] Particularly, this invention provides a DNA encoding a protein defined in (a) or (b) as described below: the DNA may be referred to as "DNA of this (the) invention" hereinbelow.

(a) A protein comprising an amino acid sequence set forth in SEQ ID NO: 2.

(b) A protein comprising an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 2 by deletion or substitution of one or more amino acids therefrom, or by addition of one or more amino acids thereto and capable of binding to nucleotide sequence CANNTG and/or nucleotide sequence CACNAG upon formation of a dimer,
wherein the amino acid sequence of a part of said protein corresponding to an amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2 is provided with not less than 85% of homology to the amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2. Preferably, DNA of this invention is a DNA defined in the following (c) or (d):

(c) A DNA comprising a nucleotide sequence of from nucleotide no. 207 to nucleotide no. 1442 of the nucleotide sequence set forth in SEQ ID NO: 1 or a complementary nucleotide sequence thereto.

(d) A DNA capable of hybridizing to the DNA defined in (c) under stringent conditions.

[0007] This invention also provides a protein encoded by DNA of the invention, as well as an antibody capable of binding to the protein.

[0008] Further, the invention provides a method for culturing human chondrocytes, which comprises monolayer culturing the chondrocytes in the presence of a membrane-permeable cAMP analog in an amount sufficient to cause the chondrocytes to maintain a differentiated state thereof as cartilage: the method may be referred to as "the culturing method of this (the) invention" hereinbelow. Preferably, the membrane-permeable cAMP analog is dibutyryl cAMP (which may be denoted "dbcAMP" hereinbelow).

[0009] Still further, the invention provides human chondrocytes that have been cultured by the culturing method of the invention and that possess the properties defined in the following (1)-(3):

(1) Exhibit a spherical shape and are abundant in extracellular matrix;
(2) Can be stained with toluidine blue satisfactorily ; and
(3) DNA of the invention is expressed therein.

**[0010]** The DNA of this invention is believed to encode a novel transcription factor of the basic helix-loop-helix type (bHLH), and is predicted to play an important role such as the regulation of expression of various genes in the differentiation of cartilage. Therefore, a DNA of this invention, a protein encoded by the DNA, and an antibody capable of binding to the protein are useful in the analysis of mechanism of the differentiation and degeneration of cartilage, as well as in the development of gene therapy for osteoarthritis and rheumatoid arthritis.

**[0011]** According to the culturing method of this invention, chondrocytes can be monolayer cultured in a good differentiated state, which makes it easy to search for genes having a distinction in expression between chondrocytes in a differentiated state thereof and chondrocytes in a dedifferentiated state thereof: namely, to search for genes expressed specifically in chondrocytes in a differentiated state thereof. Also, an analysis of the properties of chondrocytes in a differentiated state thereof will be facilitated.

## BREIF DESCRIPTION OF DRAWINGS

**[0012]**

Fig. 1 shows a comparison between DEC1 and other bHLH factors in the bHLH region.
Fig. 2A is a photomicrograph showing the morphology of human chondrocytes in monolayer culture (a-no addition of dbcAMP, b-addition of dbcAMP).
Fig. 2B is a photograph showing the morphology of human chondrocytes in monolayer culture (the morphology of an organism) that were stained with toluidine blue (a-no addition of dbcAMP, b-addition of dbcAMP).
Fig. 3 is a graph showing that DEC1 mRNA was induced in a fibroblast strain, MRC5, originating in the human lung after addition of dbcAMP.
Fig. 4 is a graph showing that DEC1 mRNA was induced in Hela cells originating in human uterine cancer after addition of dbcAMP.
Fig. 5 is a graph showing that EC1 mRNA was induced in rabbit chondrocytes after addition of PTH.
Fig. 6 is a graph showing that DEC1 mRNA was induced in a rabbit chondrocyte culture system after addition of dbcAMP.
Fig. 7 is a graph showing that DEC1 mRNA was induced in a renal cell strain after addition of dbcAMP.

## BEST MODE FOR CARRYING OUT THE INVENTION

**[0013]** Embodiments of this invention will be explained hereinbelow.

**[0014]** As will be later shown in the Examples, any genes expressed specifically in chondrocytes in a differentiated state were searched for; and as a result, the amino acid sequence set forth in SEQ ID NO: 2 has been revealed for the first time. As used in the present specification, the protein having this amino acid sequence may be referred to as "DEC1."

**[0015]** Through a homology search using a protein database, it was found that DEC1 has a basic helix-loop-helix (bHLH) region (amino acid nos. 51-108 in SEQ ID NO: 2). It is known that a bLHL protein forms a dimer and binds to E box (CANNTG).

**[0016]** Particularly, in this bHLH region, rat HES1 (47.5%), rat HES2 (42.6%), rat HES3 (40.3%), rat HES5 (37.7%), Drosophila Hairy (abbreviated as "hairy") (39.3%), and Enhancer of Split m7 (abbreviated as "E(spl)m7") (37.7%) showed high homology: numerical values in the parentheses represent homology levels. Fig. 1 shows corresponding bHLH regions of the respective proteins for comparison. The conserved residues are enclosed by the frames.

**[0017]** The HES family, hairy, and E(spl)m7 function as negative regulators which repress transcription by binding to N box (CACNAG). (Sasai et al. Genes & Dev. 6, 2620-2634 (1992); Ishibashi et al. Eur. J. Biochem. 215, 645-652 (1993); Akazawa et al. J. Biol. Chem. 267, 21879-21885 (1992); Ohsako et al. Genes & Dev. 8, 2743-2755 (1994); Dawson et al. Mol. Cell. Biol. 15, 6923-6931 (1995); Jan et al. Cell 75, 827-830 (1993).) They also have the Trp-Arg-Pro-Trp (WRPW) domain (SEQ ID NO: 3) at their C-terminus: the domain is believed to lead to suppression of a certain activator by a repressor (Dawson et al. Mol. Cell. Biol. 15, 6923-6931 (1995)). Although DEC1 resembles a bHLH factor, it does not have this WRPW domain. Thus, DEC1 is believed to be a novel transcription factor that modulates chondrogenesis. In view of the foregoing, it is thought that DEC1 is able to bind to E box, as well as to N box.

**[0018]** Accordingly, the DNA of this invention encompasses: a DNA that encodes a protein comprising the amino acid sequence set forth in SEQ ID NO: 2; and in addition, a protein comprising an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 2 by deletion or substitution of one or more amino acids therefrom,

or by addition of one or more amino acids thereto and capable of binding to nucleotide sequence CANNTG and/or nucleotide sequence CACNAG (preferably, nucleotide sequence CANNTG) upon formation of a dimer,

wherein the amino acid sequence of a part of said protein corresponding to an amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2 is provided with not less than 85% (preferably, not less than 90%) of homology to the amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2. Here, "N" in the above-described sequence represents A, G, C, or T. For an example of nucleotide sequence CANNTG, mentioned are CACGTG, CAGGTG, CAGTTG, and CACCTG. The expression, "encoding a protein," means that either one of complementary double strands has a nucleotide sequence encoding the protein when the DNA is double-stranded.

**[0019]** Substitution, deletion, or insertion of (an) amino acid residue(s) can be generated by introducing into a nucleotide sequence, variation such as the substitution, deletion, or insertion of nucleotide, according to a known method (e.g., site-specific mutation). Methods for determining the activity which allow the binding to nucleotide sequence CANNTG or CACNAG when the dimer is formed are known: for example, see Ohsako et al. Genes & Dev. 8, 2743-2755 (1994). One skilled in the art can readily select such substitution, deletion, or insertion of one or more amino acid residues that would not substantially impair this activity.

**[0020]** Concrete examples of DNA of this invention encompass DNAs defined in the following (c) and (d):

(c) A DNA comprising a nucleotide sequence of from nucleotide no. 207 to nucleotide no. 1442 of the nucleotide sequence set forth in SEQ ID NO: 1 or a complementary nucleotide sequence thereto.
(d) A DNA capable of hybridization to the DNA defined in (c) under stringent conditions.

**[0021]** Here, the "stringent conditions" means the conditions under which a so-called specific hybrid is formed, but any non-specific hybrid is not formed. These conditions may be difficult to be accurately expressed as numerical values; and for example, mentioned is a temperature in the range of from Tm to Tm minus 20 ° C where the Tm is the one for a perfectly matched hybrid, such as that between highly homologous nucleic acids, or alternatively the conditions under which DNAs having homology of not less than 80% hybridize to each other, but nucleic acids having homology lower than that do not hybridize to each other.

**[0022]** The DNA of this invention is preferably one that encodes the amino acid set forth in SEQ ID NO: 2, and is more preferably the DNA of (c) described above.

**[0023]** As will be later shown in the Examples, one of the nucleotide sequences for DNA of the invention has been determined; therefore, it is possible to synthesize the DNA based on this sequence. The DNA can also be obtained from chromosomal DNAs by PCR or hybridization using oligonucleotides or probes that have been prepared based on this very nucleotide sequence. Alternatively, the DNA can further be obtained either by carrying out RT-PCR with cartilage mRNA or by screening a cDNA library, such as cartilage, with polynucleotides having a nucleotide sequence that encodes the whole or a part of DEC1 as probes.

**[0024]** The protein encoded by DNA of this invention is a protein defined in (e) or (f) described below: the protein may be referred to as "the protein(s) of this (the) invention" hereinbelow.

(e) A protein comprising an amino acid sequence set forth in SEQ ID NO: 2.
(f) A protein comprising an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 2 by deletion or substitution of one or more amino acids therefrom, or by addition of one or more amino acids thereto and capable of binding to nucleotide sequence CANNTG and/or nucleotide sequence CACNAG upon formation of a dimer,
wherein the amino acid sequence of a part of said protein corresponding to an amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2 is provided with not less than 85% of homology to the amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2.

**[0025]** The protein of this invention can be produced by the following steps: a DNA of this invention is inserted into a known expression vector to construct a recombinant plasmid; transformed cells are obtained by introducing this recombinant plasmid thereto; the transformed cells are cultured in a suitable medium to allow a protein of the invention to form and accumulate in the culture; and said protein is harvested from the culture.

**[0026]** Host-vector systems ordinarily used to express exogenous proteins can be employed as cell and expression vectors. For example, mentioned are a combination of a prokaryotic cell such as *E. coli* and an expression vector adapted to the cell and a combination of a eukaryotic cell such as a mammalian cell and an expression vector adapted to the cell. Culture media and culturing conditions may appropriately be selected in accord with the cells to be employed.

**[0027]** The proteins of this invention may be expressed as fusion proteins with other proteins. Also, the proteins of the invention may be expressed in their full-length, or alternatively, portions thereof may be expressed as partial peptides.

[0028] Cultures, as used herein, are media and cells in the media. Harvesting proteins of this invention from the cultures can be carried out according to known protein purification methods which utilize, among others, the abov -mentioned activity of proteins of the invention as an index.

[0029] Antibodies capable of binding to the proteins of this invention can be prepared according to standard methods using the proteins of the invention as antigens: the antibodies may be referred to as "antibody (ies) of this (the) invention." The antibodies of this invention may be monoclonal antibodies or polyclonal antibodies.

[0030] The protein of this invention may be used intact as an antigen; however, it is preferred that the protein be conjugated to keyhole lymphet hemocyanine, bovine serum albumin, egg white albumin, etc. and/or be combined with adjuvants for use as the antigen.

[0031] An animal to be immunized such as a mouse, rabbit, guinea pig, or sheep is immunized by administration of the antigen described above via percutaneous, intraperitoneal, or intravenous injections or the like. A polyclonal antibody can, for example, be obtained by collecting serum from the immunized animal.

[0032] A monoclonal antibody can, for example, be obtained in the following manner. After an animal to be immunized such as a mouse, rabbit, guinea pig, or sheep is immunized by administration of the antigen described above via percutaneous, intraperitoneal, or intravenous injections or the like, its spleen or lymph node is extracted. Cells taken from this are fused with myeloma cells, which are preferably derived from the same animal species as that of the immunized animal, to create a hybridoma. A cell strain is selected by repeating screening and cloning from the obtained hybridoma: the strain incessantly produces an antibody specific to the above-mentioned antigen. A monoclonal antibody is produced in a suitable medium by culturing the thus-selected cell strain in the medium; or alternatively, it is produced in an ascite fluid or the like by culturing the strain in vivo, such as mouse abdominal cavities.

[0033] Purification methods for the resulting polyclonal and monoclonal antibodies include salting out with ammonium sulfate, ion-exchange chromatography using a DEAE cellulose column or the like, affinity chromatography using a Protein A column, and immunoabsorption chromatography. The antibodies of this invention can be detected by immunoassays using the proteins of the invention or labeled antibodies.

[0034] The antibodies of this invention may be fragmented ones, so long as they retain antigen-binding sites (Fab). Specifically mentioned as the present fragmented antibody is a fragment containing Fab that can be obtained by digesting the present antibody with a protease such as papain which does not digest the antigen-binding site.

[0035] The antibodies of this invention may be labeled by being bound to labeling substances. The labeling substances are not particularly limited, so long as they can ordinarily be used in labeling of proteins; and mentioned are enzymes, isotopes, fluorescent substances, etc.

[0036] Next, the culturing method of this invention will be explained. The method of the invention is characterized in that it comprises monolayer culture of chondrocytes in the presence of a membrane-permeable cAMP analog in an amount sufficient to cause the chondrocytes to maintain a differentiated state thereof as cartilage.

[0037] Monolayer culture of chondrocytes can be carried out in a manner similar to the conventional monolayer culture of chondrocytes, except that it is to be done in the presence of the membrane-permeable cAMP analog. For example, the media that are used for culture include α-modified Eagle's medium containing fetal bovine serum, ascorbic acid, an antibiotic, etc. as appropriate.

[0038] The membrane-permeable cAMP analog is an analog of cAMP that has the ability to permeate the membrane without impairing its function as a so-called second messenger of cAMP; preferably, it is dibutyryl cAMP.

[0039] The quantity of the membrane-permeable cAMP analog present in a medium may be such that it is sufficient to cause the chondrocytes to maintain a differentiated state thereof as cartilage. For example, in the case of dibutyryl cAMP, it is preferably 0.3-0.5 mM.

[0040] As used in the present specification, the "differentiated state" means that the chondrocytes possess at least the properties described in the following (1) to (2):

(1) Exhibit a spherical shape and are abundant in extracellular matrix; and
(2) Can be stained with toluidine blue satisfactorily.

[0041] The membrane-permeable cAMP analog in an amount sufficient to cause chondrocytes to maintain a differentiated state thereof as cartilage can also induce the differentiation of dedifferentiated chondrocytes.

[0042] Furthermore, this invention provides human chondrocytes that have been cultured by the culturing method of the invention and that possess the properties described in the following (1) to (3):

(1) Exhibit a spherical shape and are abundant in extracellular matrix;
(2) Can be stained with toluidine blue satisfactorily; and
(3) DNA of this invention is expressed therein.

[0043] Since toluidine blue selectively stains sulfated proteoglycan, the chondrocytes of this invention synthesize

the sulfated proteoglycan.

[0044] These chondrocytes express mRNAs for collagen of Type I and Type II as well as those for aggrecan.

EXAMPLES

[0045] This invention will be explained by way of examples hereinbelow.

(EXAMPLE 1) Culture of Chondrocytes in Differentiated State

[0046] Epiphyseal cartilage of the femur knee joint of a human fetus that was naturally aborted about 25 weeks of pregnancy (obtained from Norman Bethune University of Medical Sciences, Department of Pathology) was obtained. Chondrocytes were isolated from this cartilage according to the same method as that described in Shimomura et al. Calcif. Tissue Res. 19, 179-187 (1975), except that the finely cut cartilage was incubated in a α-modified Eagle's medium (α-MEM) containing 3 mg/ml collagenase (Type IA, Sigma) for 3h. The cells were seeded at 1x105 cells per Type I collagen-coated dish and maintained in α-MEM (10 ml/dish) containing 10% fetal bovine serum, 50 μg/ml of ascorbic acid, 32 unit/ml of penicillin, and 40 μg/ml of streptomycin. Dibutyryl cAMP (dbcAMP) (1 mM) was added to the culture medium when the cells became subconfluent. Cells were cultured over two days either in the presence or in the absence of dbcAMP. Then, while the cells were harvested, the morphological change of cells was examined. After fixing with ethanol, the cells were stained with toluidine blue.

[0047] The chondrocytes cultured in the presence of dbcAMP exhibited a spherical shape and were abundant in extracellular matrix, whereas the chondrocytes cultured in its absence were fibroblast-like and have a spindle shape, and were deficient in extracellular matrix. Fig. 2A shows a photomicrograph of the morphology of cells on the 6th day after addition of dbcAMP (a-no addition of dbcAMP, b-addition of dbcAMP).

[0048] In the staining with toluidine blue that should selectively stain sulfated proteoglycan, the chondrocytes cultured in the presence of dbcAMP were stained satisfactorily, whereas the chondrocytes cultured in its absence were hardly stained. Fig. 2B shows the results of staining with toluidine blue of cells on the 12th day after addition of dbcAMP (a: no addition of dbcAMP, b: addition of dbcAMP).

[0049] Expression of the mRNA for Type I and Type II collagen, and aggrecan, which serve as molecular markers, was investigated by RT-PCR. Expressions of the molecular markers were compared in the presence of dbcAMP as well as in its absence; as a result, it was indicated that the differentiated state was maintained in the presence of dbcAMP.

[0050] Accordingly, it was recognized that the chondrocytes cultured in the presence of dbcAMP maintained a differentiated state thereof as cartilage (namely, the maintenance of a differentiated phenotype).

[0051] When dose-dependence of the above-noted effect by dbcAMP was studied, said effect increased in a dose-dependent manner, reaching its maximum at 0.3-0.5 mM.

[0052] It has been reported that bFGF and TGF-β stabilize or stimulate the expression of rabbit or chicken chondrocytes of the differentiated phenotype. Employing bFGF (0.4 ng/ml) and TGF-β (3 ng/ml), the chondrocytes were cultured similarly to above, but they did not maintain their differentiated phenotype in human chondrocytes.

(EXAMPL 2) Expression of Specific Genes in the Differentiated Chondrocytes

[0053] Total RNA was extracted from the chondrocytes cultured in the presence of dbcAMP and those cultured in the absence of dbcAMP (as described in Example 1) by the guanidinethiocyanate/cesium trifluoroacetate method. Poly(A)+RNA was concentrated using Oligotex-dT30 (Roche). The subtractive hybridization was used to select clones in which mRNA was observed that had been expressed in the differentiated chondrocytes (+dbcAMP) but not in the dedifferentiated chondrocytes (-dbcAMP). Using a PCR select cDNA subtraction kit (Clonetech), cDNA synthesized from the mRNA of the differentiated chondrocytes was allowed to hybridize with an excess amount of cDNA ofrom the mRNA of the dedifferentiated chondrocytes. The cDNA that did not hybridize, namely that was expressed in a differentiated state was amplified by suppression PCR according to the manufacturer's manual. The resulting PCR product was cloned into pGEM-T (Promega), a T tail vector, and the nucleotide sequence determination was carried out on about 120 clones. One clone (pSUB37) was selected for further analysis, and the corresponding protein product was named DEC1.

[0054] NcoI-PstI fragment from pSUB37 was used as a probe to study the expression of DEC1 mRNA in various human fetus tissues by Northern blot analysis. Consequently, DEC1 was expressed in the cartilage, spleen, intestine, and lung; and it was also expressed in the heart, liver, brain, and stomach, although in small amounts. The Northern blot analysis was conducted in the following manner. Total RNA samples (5 or 10 μg) were electrophoresed on a 1% agarose gel containing formaldehyde and were transferred to Highbond-N-membranes (Amersham). Total RNA samples of various human fetus tissues were provided by Dr. Li Yu at Norman Bethune University of Medical Sciences, which were intended for the study of tissue distribution. NcoI-PstI fragment from pSUB37 was labeled with [$^{32}$P]dCTP,

and it was used as a hybridization probe. The membranes were washed with 2xSSC containing 0.5% SDS at 65 °C for 30 min. Biomax X-ray films were exposed to the washed membranes using sensitizing films at -70 °C.

**[0055]** The full-length nucleotide sequence of DEC1 cDNA was determined in the following manner. A full-length cDNA of DEC1 was isolated by RACE (rapid amplification cDNA ends method) using a Marathon cDNA amplification kit (Clonetech). Specifically, a double-stranded cDNA was ligated to a Marathon cDNA adapter and subjected to suppression PCR. Reaction was carried out using an adapter primer and a gene-specific primer that had been designed for DEC 1 based on the nucleotide sequence of pSUB37. The amplified cDNA sample was separated on a 4% polyacrylamide gel, DNA of the main band was extracted from the gel, and it was subcloned into pGEM-T. Double-stranded DNA of the subcloned plasmid and a series of synthetic oligonucleotides were used as a sequence-determining template and as a specific primer, respectively. DNA sequence determination was carried out by the Sanger method using either a sequenase 7-deaza-dGTPDNA sequencing kit (Amersham) or an ABI PRIZM 310 autosequencer (Perkin-Elmer).

**[0056]** The nucleotide sequence of DEC1 cDNA thus determined and the amino acid sequence deduced therefrom are set forth in SEQ ID NO: 1. This amino acid sequence alone is also set forth in SEQ ID NO: 2. DEC1 cDNA has an open reading frame of 1236 bp. A 2922 bp length, excluding the poly A region, is well in accord with the size of mRNA (3.1 kb) that was obtained by the Northern blot analysis described above. Since there is a stop codon in the 5'-region which serves as an inframe, the first ATG is regarded as an initiation codon. The sequence around the first ATG coincides with a Kozak consensus sequence (GCCGCCA/GCCATGG). Thus, DEC1 comprises 412 amino acids and its calculated molecular weight is 45.5 kDa.

(EXAMPLE 3)

(1) Materials and Methods

**[0057]** Chondrocytes were isolated from the rib growth plate and resting cartilage of ribs of a male Japanese white rabbit (four-weeks old) according to the method as already reported in Shimomura et al. Calcif. Tissue Res. 19, 179-187 (1975).

**[0058]** These cells were seeded at $5x10^5$ cells per 10 mm plastic culture dish and maintained in 10 ml of α-MEN supplemented with 10% FBS, 60 mg/ml of kanamycins, 250 ng/ml of amphotericin B, and 50 unit/ml of penicillin G at 37 °C in the air containing 5% $CO_2$. After cells reached confluent, the cells were washed with PBS, transferred to a fresh α-MEN (10 ml) containing no serum, and maintained for 48 h. From 1 to 24 h before the completion of incubation, 1 mM dbcAMP or $10x^{-7}$ M human recombinant PTH-(1-84) was added to the medium.

**[0059]** Human embryonic pulmonary fibroblasts (MRC-5), human cervix uteri epithelial cells (Hela), human hepatoma cells (HepG2) and canine renal epithelial cells (MDCK) were obtained from the Gene Bank of The Institute of Physical and Chemical Research. Cells were cultured in modified Dalbeco Eagle's media (DMEM) supplemented with 10% FBS until they reached confluent. After the cultures had turned confluent, the cells were washed with 10% PBS, transferred to fresh DMEM (10 ml) containing no serum, and maintained for 48 h. From 1 to 24 h before the completion of incubation, 1 mM dbcAMP was added to the media. Then, cells were harvested for RNA preparations.

(2) Northern Blot Analysis

**[0060]** Total RNAs were extracted from the cultured cells by the guanidine thiocyanate/cesium trifluoroacetate method. (Smale G. and Sasse J., Anal. Biochem. 203, 352-356 (1992).) The total RNA samples (8-20 μg) were electrophoresed on a 1% agarose gel containing formaldehyde and were transferred to NYTRAN membranes (Schleicher & Schuell, Japan). A 1.1 kb NcoI-PstI fragment from pSUB37 was labeled with [$^{32}$P]dCTP, and it was used as a hybridization probe. The membranes were washed with 0.2xSSC containing 0.5% DSD at 55 °C for 30 min. BioMax X-ray films (Eastman Kodak Co., Rochester, NY) were exposed to the washed membranes using sensitizing screens at -70 °C.

(3) Results

**[0061]**

(I) In human pulmonary fibroblast cell line MRC5, DEC1 mRNA was induced from 1 to 24 h after addition of dbcAMP (Fig. 3).
(II) In the Hela cells originating in human uterine cancer, DEC1 mRNA was also induced within 1 h after addition of dbcAMP (Fig. 4).
(III) In the rabbit chondrocytes, DEC1 mRNA was induced from 1 to 24 h after addition of PTH (Fig. 5).

(IV) In the rabbit chondrocyte culture system, it was also confirmed that DEC1 mRNA had been induced after addition of dbcAMP (Fig. 6)

(v) In the HepG2 cell strain originating in human hepatic cell line, no change in the level of DEC1 mRNA was observed between 1 to 6 h after addition of dbcAMP.

(VI) In the renal cell strain, the addition of dbcAMP also induced DEC1 mRNA (Fig. 7).

**[0062]** The above results have indicated that DEC1bHLH transcription factor is involved in the mechanism of action of PTH/PTH-rp in chondrocytes. Furthermore, the DEC1bHLH transcription factor was induced in response to the cAMP within one hour in almost all the mesenchymal and epithelial cells tested. This has suggested that the present transcription factor almost universally participates in the gene expression of the cAMP signal system.

## INDUSTRIAL APPLICABILITY

**[0063]** As has been explained above, according to this invention there are provided a gene specifically expressed in differentiated human chrondrocytes, a method for culturing human chrondrocytes in a differentiated state, and human chrondrocytes that have been cultured by the method. These are important not only in the analysis of differentiation and degeneration of cartilage, but also in the development of gene therapy for osteoarthritis and rheumatoid arthritis. Moreover, they are believed to be useful in the treatment of diseases involving other cAMP systems, because cAMP induces DEC1 mRNA in a large number of cells other than the chrondrocytes.

SEQUENCE LISTING

SEQ ID NO: 1

LENGTH: 2948

TYPE: nucleic acid

STRANDEDNESS: double-stranded

TOPOLOGY: linear

MOLECULE TYPE: cDNA

ORIGINAL SOURCE: human

CELL TYPE: chrondrocyte

FEATURES:

NAME/KEY: CDS

LOCATION: 207-1442

SEQUENCE DESCRIPTION:

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CCCCCGAAGC CCTCTCCAGG GCAGTCCTCA TCCAGACGCT CCGCTAGTGC AGACAGGAGC   180

GCGCAGTGGC CCCGGCTCGC CGCGCC ATG GAG CGG ATC CCC AGC GCG CAA CCA    233
                             Met Glu Arg Ile Pro Ser Ala Gln Pro
                               1               5

CCC CCC GCC TGC CTG CCC AAA GCA CCG GGA CTG GAG CAC GGA GAC CTA     281
Pro Pro Ala Cys Leu Pro Lys Ala Pro Gly Leu Glu His Gly Asp Leu
 10                  15                  20                  25

CCA GGG ATG TAC CCT GCC CAC ATG TAC CAA GTG TAC AAG TCA AGA CGG     329
Pro Gly Met Tyr Pro Ala His Met Tyr Gln Val Tyr Lys Ser Arg Arg
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                30                  35                  40

GGA ATA AAG CGG AGC GAG GAC AGC AAG GAG ACC TAC AAA TTG CCG CAC   377
Gly Ile Lys Arg Ser Glu Asp Ser Lys Glu Thr Tyr Lys Leu Pro His

            45                  50                  55

CGG CTC ATC GAG AAA AAG AGA CGT GAC CGG ATT AAC GAG TGC ATC GCC   425
Arg Leu Ile Glu Lys Lys Arg Arg Asp Arg Ile Asn Glu Cys Ile Ala

        60                  65                  70

CAG CTG AAG GAT CTC CTA CCC GAA CAT CTC AAA CTT ACA ACT TTG GGT   473
Gln Leu Lys Asp Leu Leu Pro Glu His Leu Lys Leu Thr Thr Leu Gly

    75                  80                  85

CAC TTG GAA AAA GCA GTG GTT CTT GAA CTT ACC TTG AAG CAT GTG AAA   521
His Leu Glu Lys Ala Val Val Leu Glu Leu Thr Leu Lys His Val Lys

90                  95                  100                 105

GCA CTA ACA AAC CTA ATT GAT CAG CAG CAG CAG AAA ATC ATT GCC CTG   569
Ala Leu Thr Asn Leu Ile Asp Gln Gln Gln Gln Lys Ile Ile Ala Leu

                110                 115                 120

CAG AGT GGT TTA CAA GCT GGT GAG CTG TCA GGG AGA AAT GTC GAA ACA   617
Gln Ser Gly Leu Gln Ala Gly Glu Leu Ser Gly Arg Asn Val Glu Thr

            125                 130                 135

GGT CAA GAG ATG TTC TGC TCA GGT TTC CAG ACA TGT GCC CGG GAG GTG   665
Gly Gln Glu Met Phe Cys Ser Gly Phe Gln Thr Cys Ala Arg Glu Val

        140                 145                 150

CTT CAG TAT CTG GCC AAG CAC GAG AAC ACT CGG GAC CTG AAG TCT TCG   713
Leu Gln Tyr Leu Ala Lys His Glu Asn Thr Arg Asp Leu Lys Ser Ser

    155                 160                 165

CAG CTT GTC ACC CAC CTC CAC CGG GTG GTC TCG GAG CTG CTG CAG GGT   761
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170                 175                 180                 185
GGT ACC TCC AGG AAG CCA TCA GAC CCA GCT CCC AAA GTG ATG GAC TTC    809
Gly Thr Ser Arg Lys Pro Ser Asp Pro Ala Pro Lys Val Met Asp Phe
                190                 195                 200
AAG GAA AAA CCC AGC TCT CCG GCC AAA GGT TCG GAA GGT CCT GGG AAA    857
Lys Glu Lys Pro Ser Ser Pro Ala Lys Gly Ser Glu Gly Pro Gly Lys
            205                 210                 215
AAC TGC GTG CCA GTC ATC CAG CGG ACT TTC GCT CAC TCG AGT GGG GAG    905
Asn Cys Val Pro Val Ile Gln Arg Thr Phe Ala His Ser Ser Gly Glu
        220                 225                 230
CAG AGC GGC AGC GAC ACG GAC ACA GAC AGT GGC TAT GGA GGA GAA TCG    953
Gln Ser Gly Ser Asp Thr Asp Thr Asp Ser Gly Tyr Gly Gly Glu Ser
    235                 240                 245
GAG AAG GGC GAC TTG CGC AGT GAG CAG CCG TGC TTC AAA AGT GAC CAC   1001
Glu Lys Gly Asp Leu Arg Ser Glu Gln Pro Cys Phe Lys Ser Asp His
250                 255                 260                 265
GGA CGC AGG TTC ACG ATG GGA GAA AGG ATC GGC GCA ATT AAG CAA GAG   1049
Gly Arg Arg Phe Thr Met Gly Glu Arg Ile Gly Ala Ile Lys Gln Glu
                270                 275                 280
TCC GAA GAA CCC CCC ACA AAA AAG AAC CGG ATG CAG CTT TCG GAT GAT   1097
Ser Glu Glu Pro Pro Thr Lys Lys Asn Arg Met Gln Leu Ser Asp Asp
            285                 290                 295
GAA GGC CAT TTC ACT AGC AGT GAC CTG ATC AGC TCC CCG TTC CTG GGC   1145
Glu Gly His Phe Thr Ser Ser Asp Leu Ile Ser Ser Pro Phe Leu Gly
        300                 305                 310
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CCA CAC CCA CAC CAG CCT CCT TTC TGC CTG CCC TTC TAC CTG ATC CCA   1193
Pro His Pro His Gln Pro Pro Phe Cys Leu Pro Phe Tyr Leu Ile Pro
   315              320                 325

CCT TCA GCG ACT GCC TAC CTG CCC ATG CTG GAG AAG TGC TGG TAT CCC   1241
Pro Ser Ala Thr Ala Tyr Leu Pro Met Leu Glu Lys Cys Trp Tyr Pro
330                 335                 340                 345

ACC TCA GTG CCA GTG CTA TAC CCA GGC CTC AAC GCC TCT GCC GCA GCC   1289
Thr Ser Val Pro Val Leu Tyr Pro Gly Leu Asn Ala Ser Ala Ala Ala
                350                 355                 360

CTC TCT AGC TTC ATG AAC CCA GAC AAG ATC TCG GCT CCC TTG CTC ATG   1337
Leu Ser Ser Phe Met Asn Pro Asp Lys Ile Ser Ala Pro Leu Leu Met
        365                 370                 375

CCC CAG AGA CTC CCT TCT CCC TTG CCA GCT CAT CCG TCC GTC GAC TCT   1385
Pro Gln Arg Leu Pro Ser Pro Leu Pro Ala His Pro Ser Val Asp Ser
    380                 385                 390

TCT GTC TTG CTC CAA GCT CTG AAG CCA ATC CCC CCT TTA AAC TTA GAA   1433
Ser Val Leu Leu Gln Ala Leu Lys Pro Ile Pro Pro Leu Asn Leu Glu
395                 400                 405

ACC AAA GAC TAAACTCTCT AGGGGATCCT GCTGCTTTGC TTTCCTTCCT          1482
Thr Lys Asp
410

CGCTACTTCC TAAAAAGCAA CAAAAAAGTT TTTGTGAATG CTGCAAGATT GTTGCATTGT  1542

GTATACTGAG ATAATCTGAG GCATGGAGAG CAGATTCAGG GTGTGTGTGT GTGTGTGTGT  1602

GTGTGTGTGT ATGTGCGTGT GCGTGCACAT GTGTGCCTGC GTGTTGGTAT AGGACTTTAA  1662

AGCTCCTTTT GGCATAGGGA AGTCACGAAG GATTGCTTGA CATCAGGAGA CTTGGGGGGG  1722

ATTGTAGCAG ACGTCTGGGC TTTTCCCCAC CCAGAGAATA GCCCCCTTCG ATACACATCA  1782
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GCTGGATTTT CAAAAGCTTC AAAGTCTTGG TCTGTGAGTC ACTCTTCAGT TTGGGAGCTG 1842

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ACCAGCTGTT AGGACCCCAC CCTTTTGCCT TTATTGTCGA CGTGACTCAC CAGACGTCGG 1962

GGAGAGAGAG CAGTCAGACC GAGCTTTCTG CTAACATGGG GAGGTAGCAG GCACTGGCAT 2022

AGCACGGTAG TGGTTTGGGG AGGTTTCCGC AGGTCTGCTC CCCACCCCTG CCTCGGAAGA 2082

ATAAAGAGAA TGTAGTTCCC TACTCAGGCT TTCGTAGTGA TTAGCTTACT AAGGAACTGA 2142

AAATGGGCCC CTTGTACAAG CTGAGCTGCC CCGGAGGGAG GGAGGAGTTC CCTGGGCTTC 2202

TGGCACCTGT TTCTAGGCCT AACCATTAGT ACTTACTGTG CAGGGAACCA AACCAAGGTC 2262

TGAGAAATGC GGACACCCCG AGCGAGCACC CCAAAGTGCA CAAAGCTGAG TAAAAAGCTG 2322

CCCCCTTCAA ACAGAACTAG ACTCAGTTTT CAATTCCATC CTAAAACTCC TTTTAACCAA 2382

GCTTAGCTTC TCAAAGGCCT AACCAAGCCT TGGCACCGCC AGATCCTTTC TGTAGGCTAA 2442

TTCCTCTTGC CCAACGGCAT ATGGAGTGTC CTTATTGCTA AAAAGGATTC CGTCTCCTTC 2502

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CACAGTGAGA AAAAATGAAT GTATTCCTGT TTTTGAAGAG AAGAATAATT TTTTTTCTC 2802

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TTTTATCTAT GAGTAAATGT TAAGTAGTTG TTTTAAAATA CTTAATAAAA TAATTCTTTT 2922

CCTGTGGAAG AAAAAAAAAA AAAAAA 2948

SEQ ID NO: 2

LENGTH: 412

TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

SEQUENCE DESCRIPTION:

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Met Glu Arg Ile Pro Ser Ala Gln Pro Pro Pro Ala Cys Leu Pro Lys
 1               5                  10                  15
Ala Pro Gly Leu Glu His Gly Asp Leu Pro Gly Met Tyr Pro Ala His
            20                  25                  30
Met Tyr Gln Val Tyr Lys Ser Arg Arg Gly Ile Lys Arg Ser Glu Asp
        35                  40                  45
Ser Lys Glu Thr Tyr Lys Leu Pro His Arg Leu Ile Glu Lys Lys Arg
    50                  55                  60
Arg Asp Arg Ile Asn Glu Cys Ile Ala Gln Leu Lys Asp Leu Leu Pro
65                  70                  75                  80
Glu His Leu Lys Leu Thr Thr Leu Gly His Leu Glu Lys Ala Val Val
                85                  90                  95
Leu Glu Leu Thr Leu Lys His Val Lys Ala Leu Thr Asn Leu Ile Asp
            100                 105                 110
Gln Gln Gln Gln Lys Ile Ile Ala Leu Gln Ser Gly Leu Gln Ala Gly
        115                 120                 125
Glu Leu Ser Gly Arg Asn Val Glu Thr Gly Gln Glu Met Phe Cys Ser
    130                 135                 140
Gly Phe Gln Thr Cys Ala Arg Glu Val Leu Gln Tyr Leu Ala Lys His
145                 150                 155                 160
Glu Asn Thr Arg Asp Leu Lys Ser Ser Gln Leu Val Thr His Leu His
                165                 170                 175
Arg Val Val Ser Glu Leu Leu Gln Gly Gly Thr Ser Arg Lys Pro Ser
            180                 185                 190
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Asp Pro Ala Pro Lys Val Met Asp Phe Lys Glu Lys Pro Ser Ser Pro
        195                 200                 205
Ala Lys Gly Ser Glu Gly Pro Gly Lys Asn Cys Val Pro Val Ile Gln
    210                 215                 220
Arg Thr Phe Ala His Ser Ser Gly Glu Gln Ser Gly Ser Asp Thr Asp
225                 230                 235                 240
Thr Asp Ser Gly Tyr Gly Gly Glu Ser Glu Lys Gly Asp Leu Arg Ser
                245                 250                 255
Glu Gln Pro Cys Phe Lys Ser Asp His Gly Arg Arg Phe Thr Met Gly
            260                 265                 270
Glu Arg Ile Gly Ala Ile Lys Gln Glu Ser Glu Glu Pro Pro Thr Lys
        275                 280                 285
Lys Asn Arg Met Gln Leu Ser Asp Asp Glu Gly His Phe Thr Ser Ser
    290                 295                 300
Asp Leu Ile Ser Ser Pro Phe Leu Gly Pro His Pro His Gln Pro Pro
305                 310                 315                 320
Phe Cys Leu Pro Phe Tyr Leu Ile Pro Pro Ser Ala Thr Ala Tyr Leu
                325                 330                 335
Pro Met Leu Glu Lys Cys Trp Tyr Pro Thr Ser Val Pro Val Leu Tyr
            340                 345                 350
Pro Gly Leu Asn Ala Ser Ala Ala Ala Leu Ser Ser Phe Met Asn Pro
        355                 360                 365
Asp Lys Ile Ser Ala Pro Leu Leu Met Pro Gln Arg Leu Pro Ser Pro
    370                 375                 380
Leu Pro Ala His Pro Ser Val Asp Ser Ser Val Leu Leu Gln Ala Leu
385                 390                 395                 400
```

Lys Pro Ile Pro Pro L u Asn Leu Glu Thr Lys Asp

405 410

SEQ ID NO: 3

LENGTH: 4

TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE DESCRIPTION:

Trp Arg Pro Trp

1

## Claims

1. A DNA encoding a protein defined in the following (a) or (b):

   (a) a protein comprising the amino acid sequence set forth in SEQ ID NO: 2;
   (b) a protein comprising an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 2 by deletion or substitution of one or more amino acids therefrom, or by addition of one or more amino acids and capable of binding to nucleotide sequence CANNTG and/or nucleotide sequence CACNAG upon formation of a dimer,
   wherein the amino acid sequence of a part of said protein corresponding to an amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2 is provided with not less than 85% of homology to the amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2.

2. A DNA represented by the following (c) or (d):

   (c) a DNA comprising a nucleotide sequence of from nucleotide no. 207 to nucleotide no. 1442 of the nucleotide sequence set forth in SEQ ID NO: 1 or a complementary nucleotide sequence thereto;
   (d) a DNA capable of hybridization to the DNA defined in (c) under stringent conditions.

3. A protein represented by the following (e) or (f):

   (e) a protein comprising the amino acid sequence set forth in SEQ ID NO: 2;
   (f) a protein comprising an amino acid sequence derived from the amino acid sequence set forth in SEQ ID NO: 2 by deletion or substitution of one or more amino acids therefrom, or by addition of one or more amino acids thereto and capable of binding to nucleotide sequence CANNTG and/or nucleotide sequence CACNAG upon formation of a dimer,
   wherein the amino acid sequence of a part of said protein corresponding to an amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2 is provided with not less than 85% of homology to the amino acid sequence of from amino acid no. 51 to amino acid no. 108 in SEQ ID NO: 2.

4. An antibody capable of binding to the protein according to claim 3.

5. A method for culturing human chondrocytes, which comprises monolayer-culturing the chondrocytes in the presence of a membrane-permeable cAMP analog in an amount sufficient to cause the chondrocytes to maintain a differentiated state thereof as cartilage.

6. The method for culturing according to claim 5, wherein the membrane-permeable cAMP analog is dibutyryl cAMP.

7. Human chondrocytes that have been cultured by the culturing method according to claim 5 or claim 6, possessing the properties defined in the following (1)-(3):

(1) exhibiting a spherical shape and being abundant in extracellular matrix;
(2) capable of being satisfactorily stained with toluidine blue; and
(3) causing the DNA according to claim 1 to be expressed therein.

Fig.1

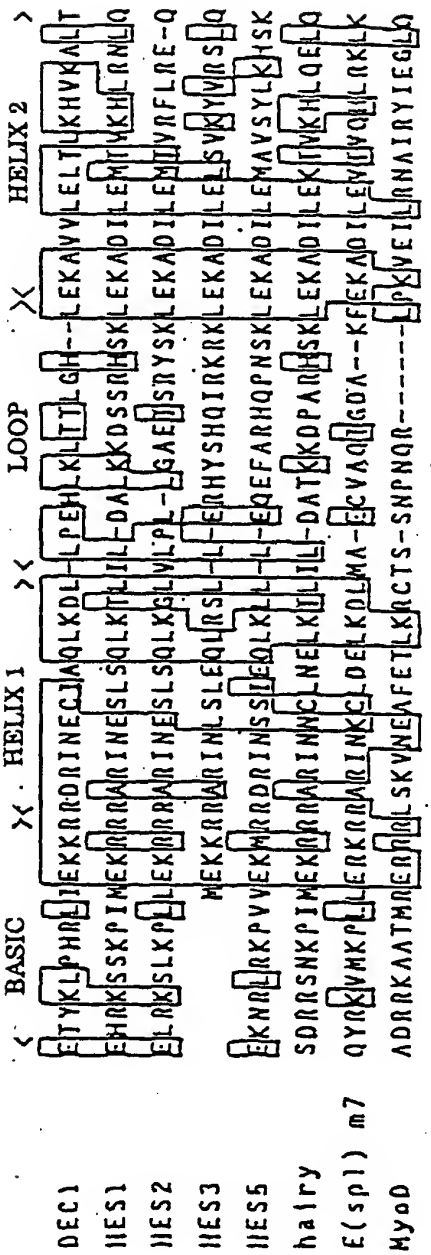

BASIC
HELIX 1
LOOP
HELIX 2
DEC1
HES1
HES2
HES3
HES5
hairy
E(spl) m7
HyoD

Fig.2A

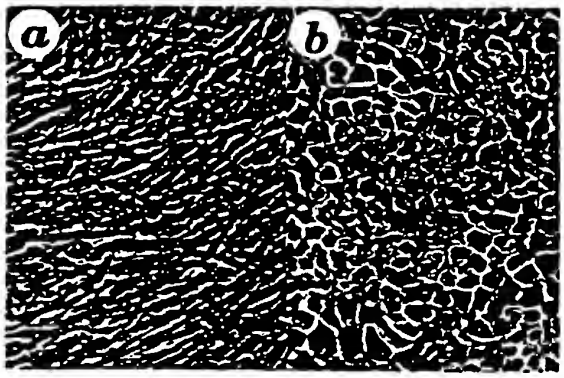


Fig.2B

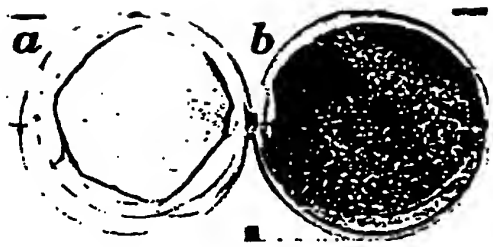


*Fig.3*

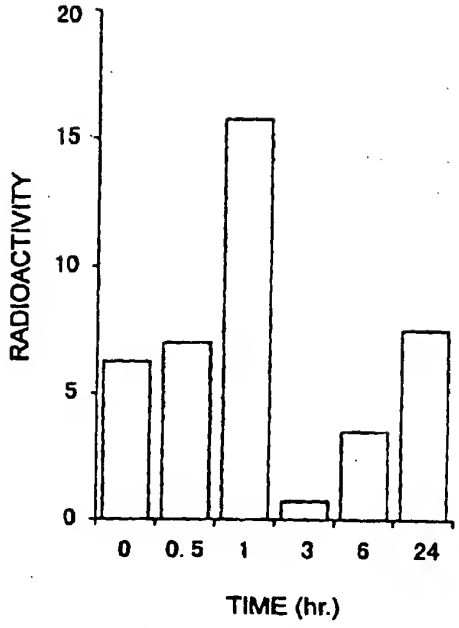


*Fig.4*

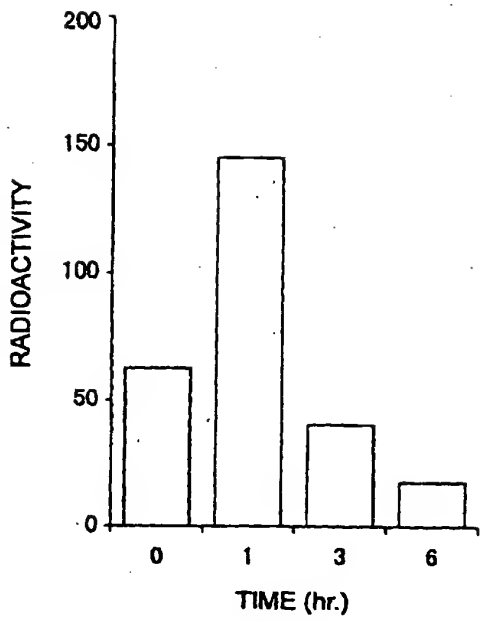


*Fig.5*

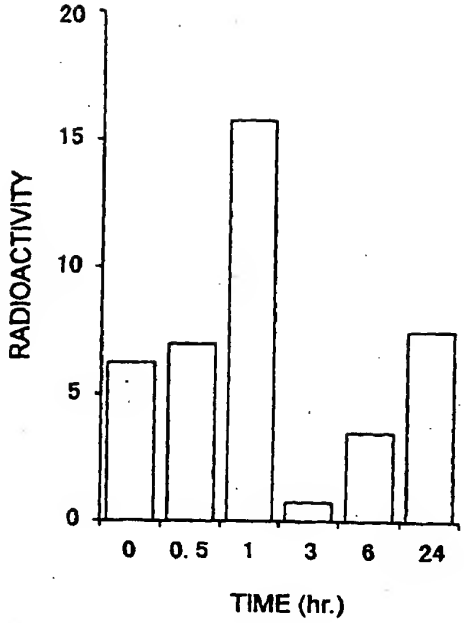


*Fig.6*

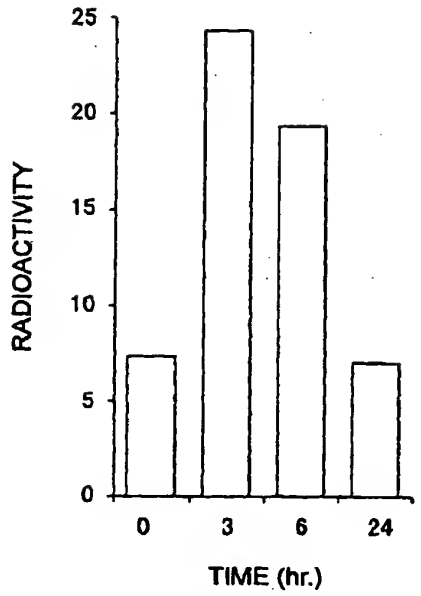
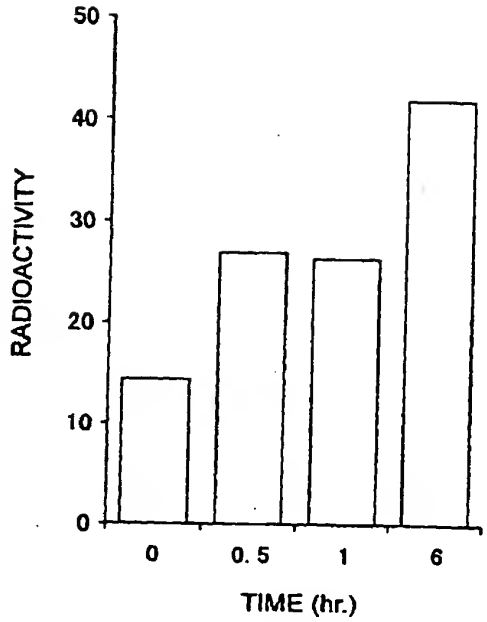


*Fig.7*



## INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/03106

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] C12N15/12, C12P21/02, C12N5/08, C07K14/47, C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] C12N15/12, C12P21/02, C12N5/08, C07K14/47, C07K16/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Genbank/EMBL/DDBJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| PX | Biochem. Biophys. Res. Commun., 236, (1997), Ming Shen et al., "Molecular characterization of the novel basic helix-loop-helix protein DEC1 expressed in differentiated human embryo chondrocytes" p.294-298 | 1-7 |
| X | WO, 96-39427, A (Trustees of dartmouth colleg), 12 December, 1996 (12. 12. 96) & EP, 832117, A | 1-4 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 28 October, 1998 (28. 10. 98) | 10 November, 1998 (10. 11. 98) |
| Name and mailing address of the ISA/ Japanese Patent Office | Authorized officer |
| Facsimile No. | Telephone No. |

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